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Effect of pH on the photoreaction cycles of bacteriorhodopsin

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Photoreaction cycles of acidified bacteriorhodopsin (bR_{605}^{acid}) at room temperature was studied by nanosecond and picosecond spectroscopy. A precursor of bathobacteriorhodopsin was found to be converted to bathobacteriorhodopsin within 30 ps. Spectra and formation times of batho- and lumibacteriorhodopsin are identical to those of bacteriorhodopsin in neutral suspension. However, lumibacteriorhodopsin lifetime is $>700\ \mu s$ and the formation of metabacteriorhodopsin was not observed in low pH suspension. Thus, the decay process of lumibacteriorhodopsin plays an important role in the proton pump action.

Bacteriorhodopsin *Photocycle* *Picosecond laser photolysis* *Nanosecond laser photolysis* *Proton pumping* *Absorption spectrum*

1. INTRODUCTION

The photochemical cycle of the light- and dark-adapted (bR_{568} and bR_{558}) purple membranes in *Halobacterium halobium* have been broadly studied [1–4]. The cycle of bR_{568} contains at least 4 intermediates which are called batho- (K_{590}), lumi- (L_{550}), metabacteriorhodopsin (M_{412}), and O_{640} [2]. Another intermediate, N_{520}, is not yet established. A Schiff base is believed to be unprotonated in the M_{412} intermediate [5].

The purple membrane changes its absorption spectrum in the visible region at low pH [1]. Absorption maximum shifts from 568 nm to 605 nm at pH 2.5 [1]. In [6] laser flash photolysis with 80 μs time resolution was performed on the acidified purple membrane called bR_{605}^{acid} and bR_{565}; these results are complicated because of limited time resolution and excitation wavelengths (588 nm and 580 nm). Pulse widths of their excitation light sources are short enough to study primary processes of bacteriorhodopsin but the time-resolution in [6] is about 80 μs. Samples were excited using 630 nm light pulse but transient spectra data were not shown in [6]; all spectra were measured using 580 and 588 nm excitation light pulse [6]. Here, we have undertaken picosecond and nanosecond spectroscopy at 30 ps and 10 ns time resolution; we have obtained a very clear pH effect on the photocycle of acidified purple membrane (bR_{605}^{acid}).

2. MATERIALS AND METHODS

The purple membrane was purified from *Halobacterium halobium* R_1M_1 as in [7]. The purified purple membranes were checked by SDS–polyacrylamide gel electrophoresis.

The purified purple membrane samples were washed with distilled water several times and treated on an ion-exchange resin column (acidic form of Dowex 50W) to remove cations. We found transparent and stable suspensions of bR_{605}^{acid} which had red-shifted absorption maxima. The formation of acidified purple membrane is reversible; bR_{605}^{acid} reverts to bR_{568} by the addition of bases or salts. The addition reduces the concentration of protons attached to the membrane. This reduction induces the deprotonation of protonated bacteriorhodopsin.

An absorption maximum of bR_{605}^{acid} is located at 603–605 nm when an absorption spectrum was

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measured just after a sample was prepared. The absorption maximum shifts slowly to the shorter wavelength region. This change is probably due to change in pH. Small amount of ion-exchange gel was added to the bottom of a measurement cell to protect the change of samples. Measurements were done <24 h after the preparation of acidified samples.

Nanosecond and picosecond absorption spectroscopy were performed with Q-switched and mode-locked Nd:YAG lasers. The second harmonics of the lasers were focused into cells containing acetone to generate the first Stokes of stimulated Raman scattering at 630 nm with 5 ns (Q-switch YAG laser-pumped) or 20 ps (mode-locked YAG laser-pumped) pulse width. The energy of the excitation pulses is about 90 μJ (ns pulse) or 76–90 μJ (ps pulse). A photomultiplier and monochromator was used for the measurement of ns time-resolved absorption spectra. Two coupled polychromator and multichannel photodiode (MCPD: Union Giken) systems were used for the measurement of ps time-resolved absorption spectra. Details of the experimental system are given in [8]; measurements were performed at 20–22°C.

3. RESULTS AND DISCUSSION

Fig.1a,b show the transient difference absorption spectra of bR_{605}^{acid} 21 ps and 217 ps after excitation by 20 ps pulse at 630 nm. Absorbance change at 630 nm is affected by the scattering of the intense picosecond excitation light. A bleaching was observed 21 ps after excitation. Increasing the delay time the shape of difference spectra continuously changed. A new absorption band appeared in the longer wavelength region and an isosbestic point shifted to the shorter wavelength. The spectral shape does not change within experimental error during the delay time between 217 ps and 100 ns.

Fig.2 shows the transient difference absorption spectra of bR_{605}^{acid} 100 ns, 14 μs and 50 μs after excitation (5 ns, 630 nm).

The transient difference spectrum 100 ns after excitation (curve 1) shows the generation of the red-shifted species which have been observed for ordinary bacteriorhodopsin bR_{568} in purple membrane in neutral (pH 7) suspension [2]. Absorbance

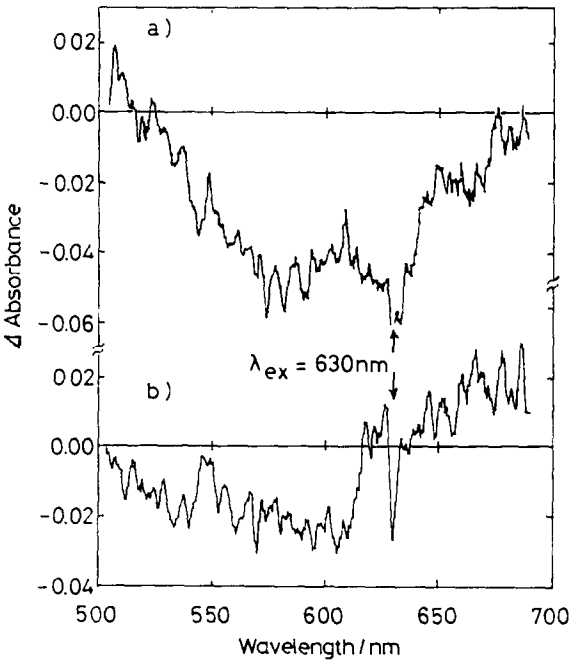

Fig.1. Picosecond time-resolved difference absorption spectra of bR_{605} 21 ps (a) and 217 ps (b) after excitation at 630 nm.

change maxima are located at 670–680 nm ($\Delta A > 0$) and 580–600 nm ($\Delta A < 0$) in low pH suspensions and 640 nm ($\Delta A > 0$) and 550 nm ($\Delta A < 0$) in neutral suspensions [2]. An isosbestic point is located at 630 nm in low pH sample and at 590 nm in neutral suspension [2]. The wavelengths of absorption maxima and isosbestic points in low pH samples are shifted to the red region by 30–50 nm which is close to the shift of the initial bacteriorhodopsin caused by low pH. These facts indicate the existence of the red-shifted intermediate in both neutral and acidic bacteriorhodopsins. The lifetime of this intermediate in acidified membrane in low pH suspension was measured as 1.5 ± 0.4 μs by observing the time dependence of the absorbance. This lifetime agrees well with that of bathobacteriorhodopsin in purple membrane in neutral suspension (1.3 ± 0.3 μs [9]). The formation time constant of the red-shifted species is estimated to be <30 ps. The time-resolution of our picosecond apparatus is ~30 ps and this result is consistent with the formation time of bathobacteriorhodopsin in purple membrane at 11 ps [10].

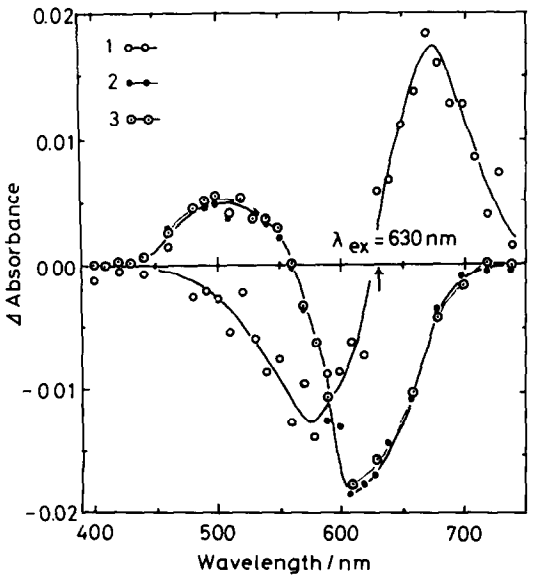

Fig.2. Nanosecond time-resolved difference absorption spectra of bR_{605}. (1–3) Transient spectra 100 ns, 14 μs and 50 μs, respectively after excitation at 630 nm.

The above results indicate that the formation of bathobacteriorhodopsin in acidified membrane in low pH suspension takes place with a <30 ps rise-time constant and decays within 1.5 ± 0.4 μs. The rise and decay time constants are the same as those of bR_{568} in purple membrane. This means that the formation and decay kinetics of bathobacteriorhodopsin are not affected by lowering pH.

The transient difference spectrum of a low pH sample observed at 50 μs is very similar to that at 14 μs delay (fig.2 (2,3)). The maxima of the absolute value of absorbance change are located at 610 nm ($\Delta A < 0$) and 500 nm ($\Delta A > 0$) with an isosbestic point at 560 nm. From the data in [2] (fig.6), the corresponding wavelengths of the difference spectrum between bathobacteriorhodopsin (K intermediate) and lumibacteriorhodopsin (L intermediate) in neutral pH suspension are calculated to be 570 nm, 450 nm and 520 nm, respectively [2]. The wavelengths observed here for low pH sample are again red-shifted by 40–50 nm from those of neutral sample. This fact means that lumibacteriorhodopsin in low pH suspension also has a similar absorption spectrum to that in neutral suspension. The temporal behavior of lumibacteriorhodopsin in low pH suspension is different from that in neutral pH suspension.

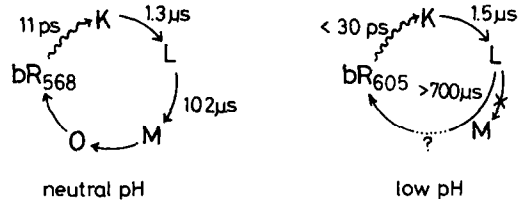

Fig.3. Reaction cycles of bacteriorhodopsin at pH 7 and low pH.

The decay time of lumibacteriorhodopsin is 102 ± 12 μs [11] in neutral pH suspension while it is longer than 700 μs in low pH suspension.

The extraordinary reduction of rate constant of the lumi- ⟶ metabacteriorhodopsin conversion in low pH suspension can be interpreted as follows:

Lumi- ⟶ metabacteriorhodopsin conversion process is considered to be proton release from the protonated Schiff base of retinal to some proton-accepting sites in neutral condition. In bR_{605}^{acid}, many of these sites are considered to be already occupied by protons in the ground state of bR_{605}^{acid}. This is thought to be the reason of red-shift of absorption maximum from 568–605 nm. Proton transfer is restricted from these occupied sites. This makes the lumi- ⟶ metabacteriorhodopsin conversion process difficult and makes the rate reduced by a factor of >7.

This result indicates that deprotonation of the chromophore takes place in the lumi- ⟶ metabacteriorhodopsin conversion process. The photoreaction cycles of bacteriorhodopsin at pH 7 and low pH are shown in fig.3.

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